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Abstract: Antiviral CD8+ T cells are a key component of the adaptive immune response against HCV, but their impact on viral control is influenced by preexisting viral variants in important target epitopes and the development of viral escape mutations. Immunodominant epitopes highly conserved across genotypes therefore are attractive for T cell based prophylactic vaccines. Here, we characterized the CD8+ T cell response against the highly conserved HLA-B*51-restricted epitope IPFYGKAI1373–1380 located in the helicase domain of NS3 in people who inject drugs (PWID) exposed predominantly to HCV genotypes 1a and 3a. Despite this epitope being conserved in both genotypes, the corresponding CD8+ T cell response was detected only in PWID infected with genotype 3a and HCV-RNA negative PWID, but not in PWID infected with genotype 1a. In genotype 3a, the detection of strong CD8+ T cell responses was associated with epitope variants in the autologous virus consistent with immune escape. Analysis of viral sequences from multiple cohorts confirmed HLA-B*51-associated escape mutations inside the epitope in genotype 3a, but not in genotype 1a. Here, a distinct substitution in the N-terminal flanking region located 5 residues upstream of the epitope (S1368P; $P = 0.00002$) was selected in HLA-B*51-positive individuals. Functional assays revealed that the S1368P substitution impaired recognition of target cells presenting the endogenously processed epitope. The results highlight that, despite an epitope being highly conserved between two genotypes, there are major differences in the selected viral escape pathways and the corresponding T cell responses. **IMPORTANCE** HCV is able to evolutionary adapt to CD8+ T cell immune pressure in multiple ways. Beyond selection of mutations inside targeted epitopes, this study demonstrates that HCV inhibits epitope processing by modification of the epitope flanking region under T cell immune pressure. Selection of a substitution five amino acids upstream of the epitope underlines that efficient antigen presentation strongly depends on its larger sequence context and that blocking of the multistep process of antigen processing by mutation is exploited also by HCV. The pathways to mutational escape of HCV are to some extent predictable but are distinct in different genotypes. Importantly, the selected escape pathway of HCV may have consequences for the destiny of antigen-specific CD8+ T cells.

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**Distinct escape pathway by HCV genotype 1a from a dominant CD8⁺ T cell
response by selection of altered epitope processing**

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Abstract:

Antiviral CD8⁺ T cells are a key component of the adaptive immune response against HCV, but their impact on viral control is influenced by pre-existing viral variants in important target epitopes and the development of viral escape mutations. Immunodominant epitopes highly conserved across genotypes therefore are attractive for T cell based prophylactic vaccines. Here, we characterized the CD8⁺ T cell response against the highly conserved HLA-B*51-restricted epitope IPFYGKAI₁₃₇₃₋₁₃₈₀ located in the helicase domain of NS3 in people who inject drugs (PWID) exposed predominantly to HCV genotypes 1a and 3a. Despite this epitope being conserved in both genotypes, the corresponding CD8⁺ T cell response was detected only in PWID infected with genotype 3a and HCV-RNA negative PWID, but not in PWID infected with genotype 1a. In genotype 3a, detection of strong CD8⁺ T cell responses was associated with epitope variants in the autologous virus consistent with immune escape. Analysis of viral sequences from multiple cohorts confirmed HLA-B*51-associated escape mutations inside the epitope in genotype 3a, but not in genotype 1a. Here, a distinct substitution in the N-terminal flanking region located 5 residues up-stream of the epitope (S1368P; p=0.00002) was selected in HLA-B*51-positive individuals. Functional assays revealed that the S1368P substitution impaired recognition of target cells presenting the endogenously processed epitope. The results highlight that, despite an epitope being highly conserved between two genotypes, there are major differences in the selected viral escape pathways and the corresponding T cell responses.

Importance: HCV is able to evolutionary adapt to CD8⁺ T cell immune pressure in multiple ways. Beyond selection of mutations inside targeted epitopes this study demonstrates that HCV inhibits epitope processing by modification of the epitope flanking region under T cell immune pressure. Selection of a substitution five amino acids upstream of the epitope underlines that efficient antigen presentation strongly depends on its larger sequence context and that blocking of

54 the multi-step process of antigen processing by mutation is exploited also by HCV. The pathways
55 to mutational escape of HCV are to some extent predictable but are distinct in different
56 genotypes. Importantly, the selected escape pathway of HCV may have consequences for the
57 destiny of antigen-specific CD8⁺ T cells.

Introduction:

Based on phylogenetic analysis HCV can be classified into at least seven genotypes and multiple subtypes that differ up to 20% at the amino acid level (1). The HCV genotypes have distinct epidemiological characteristics, are associated with different transmission risk factors and their frequencies in a population are regionally different. In Europe and North America the HCV genotypes 1 and 3 are most common (2, 3). Since routine screening via nucleic acid amplification for HCV eliminated the risk for infection through blood products, the most important risk group for incident HCV infection are people who inject drugs (PWID). The high prevalence of HCV infection with seroprevalence rates up to 80% paired with frequent risk practices for HCV transmissions in PWID results in incidence rates between 8% and 25% per year in young adult injectors (4, 5) and there is strong evidence that multiple exposures are common in this risk group (6, 7). The degree of sequence diversity between genotypes and subtypes precludes broad protection against re-infection. Accordingly, multiple infections of the same individual with different viruses have been reported (7).

Even at the subtype level HCV isolates typically differ between hosts. Lack of a proof reading function of the virus encoded RNA-dependent RNA polymerase results in a high error rate during RNA replication. As a consequence, HCV exists in chronically infected patients as a quasispecies of closely related but genetically distinct viral variants. There is now strong evidence that viral genetic variation between hosts is the product of continuous selection of mutations by host immune pressure (8-14). Collectively, this inherent sequence diversity of HCV at the genotype, subtype and quasispecies level is a major obstacle to vaccine design (15). Strategies that aim to develop prophylactic vaccines against HCV have to cope with this genetic heterogeneity either by inducing immune responses with a high degree of cross-reactivity (16, 17), by inducing multiple

responses against different sequence variants (18) or by focussing the immune response on highly conserved regions of the virus.

Dominant CD8⁺ T cell epitopes that are conserved across different HCV genotypes are rare (19, 20). Here, we characterized a highly conserved dominant HLA-B*51-restricted CD8⁺ T cell epitope (IPFYGKAI₁₃₇₃₋₁₃₈₀) in HCV NS3 in PWID predominantly exposed to genotype 1a and 3a. Vigorous responses were detected in PWID with spontaneous immune control of HCV and in PWID with genotype 3a infection, but not in PWID infected with genotype 1a. Although selection of mutations inside the epitope was overall rare, there was evidence for mutational escape in HCV genotype 1b and 3a by population sequence analysis. Interestingly, HCV genotype 1a followed a distinct escape pathway by selecting a substitution (S1368P) located five amino acids up-stream of the epitope. Further analysis of the functional relevance revealed that the S1368P substitution altered epitope processing. The results demonstrate that beyond selection of mutations inside CD8⁺ epitopes HCV adapts to immune pressure by selecting mutations in the epitope flanking region. The evolutionary escape pathways differ between HCV genotypes indicating distinct genetic plasticity.

Methods:

Patients

Blood samples from patients with a history of injection drug use were collected from the ward for inpatient detoxification treatment of drug addicts or the clinic for opioid maintenance treatment (OMT) at the Department of Addictive Behaviour and Addiction Medicine, Rhine State Hospital Essen, Hospital of the University of Duisburg-Essen. Written informed consent was obtained from all study participants and the study was approved by the ethics committee of the Medical

Faculty of the University of Duisburg-Essen in accordance with the Declaration of Helsinki. In total 43 HLA-B*51 positive treatment-naïve subjects who were HCV antibody positive (by anti-HCV CMIA from Abbott) were analyzed, including 15 HCV-RNA negative patients and 28 subjects with detectable HCV-RNA. Peripheral blood mononuclear cells (PBMCs) were isolated from blood via Ficoll gradient centrifugation and subsequently cryopreserved.

Analysis and Alignment of HCV Sequences

The frequency of HLA-B*51-associated sequence polymorphisms was analyzed in 405 HCV genotype 1a sequences and 145 HCV genotype 1b sequences from a multi-center cohort (21) and in a large HCV genotype 1b outbreak (22). A region covering the epitope IPFYGKAI₁₃₇₃₋₁₃₈₀ was amplified and sequenced from additional 37 HCV genotype 1a isolates as well as 102 HCV genotype 3a isolates collected in Germany. All sequences have been submitted to GenBank (accession no. FJ864775-864816, KJ130249-130317 and KJ668232-KJ668268).

Analysis of the CD8⁺ T Cell Response

Antigen-specific T cells were expanded from cryopreserved HLA-B*51 positive PBMCs utilizing synthetic peptides (>70% purity) purchased from EMC, Tübingen, Germany. After thawing PBMCs were cultured in RPMI 1640 medium containing 10% fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 10 mM HEPES buffer and 25 U/ml recombinant interleukin-2 (IL-2) and stimulated with the HLA-B*51 peptide 1373 (1 µg/ml per peptide) and 0.1 µg/ml anti-CD28 and anti-CD49d. After 7 days medium containing IL-2 was added. On day 10 the cells were restimulated with the same peptide (10 µg/ml) in the presence of Brefeldin A (100 ng/ml) for 4 h and then analysed for their CD4⁺, CD8⁺ and IFN-γ expression via flow cytometry. To determine the degree of cross-reactivity between different B*51-1373 variants,

PBMCs were cultivated in the presence of the HLA-B*51-1373 prototype or the variant peptide. After 10 days, both cultures were restimulated with the prototype and the variant peptide at different concentrations before intracellular IFN- γ staining. For detection of HCV-specific cells *ex vivo* and after peptide-specific *in vitro* expansion, thawed PBMCs were stained with PE-labeled IPFYGKAI-specific HLA-B*5101 dextramer (Immudex, Denmark) followed by surface staining with CD8⁺ PerCP-Cy5.5 (eBioscience). All samples were acquired using a FACS Canto (BD) and the data was analyzed using FlowJo software (Tree Star Inc.)

Expression plasmids for HCV-GFP fusion proteins

All constructs were based on the parental plasmids pEGFP-N1 (Clontech, Germany) featuring a CMV promoter controlled eGFP protein for N-terminal fusion. Fragments of NS3 were generated by amplifying NS3 1329-1433 by nested PCR with primers 4b-F (CCTACGGCAAGTTCCTTGC) and 4b-R_{new} (GCAGTCTATCACCGAGTCG) and B51-for-EcoRI (ccaaggggaattcttggtctctcaccctcgccatcgccacgcygtccttgaccaag) and B51-Rev-Sall (cggataccgtcgacccgcggtartamgccacggc). Subsequently the fragment was cloned via EcoRI/Sall into pEGFP-N1. The substitution S1368P was introduced via site directed mutagenesis (Stratagene) using primer B51-S1368P-for (ggaggttgctctgcccaccaccggaga) and B51-S1368P-rev (tctccggtggtgggcagagcaacctcc) according to the manufacturer's instructions. All constructs were verified by DNA sequencing.

Analysis of the CD8⁺ T cell response against endogenously processed antigens

HLA-B*51 PBMCs from healthy donors were obtained from buffy coats (Department of Transfusion Medicine, Essen). For analysis of endogenously processed antigens, HLA-B*51 positive cells were electroporated with plasmids pac-NS3-S1368S-GFP or pac-NS3-S1368P-GFP

encoding a NS3-GFP fusion protein utilizing the Amaxa T cell nucleofactor kit (vpa-1002; Lonza). In brief, after thawing PBMCs were cultured in RPMI medium. After 24 h 7×10^6 cells were re-suspended in 100 μ l Nucleofactor™ Solution, mixed with 7.5 μ g DNA and pulsed with the Optimized Protocol for Unstimulated Human T Cells (Program v024, Amaxa2b) with an Amaxa apparatus (Lonza). After electroporation PBMCs were cultured in RPMI medium for 24 h cells before GFP positive cells were sorted using a FACS Aria II (BD). Subsequently, 1×10^5 B*51-1373 specific cells from a 10 day culture were restimulated with GFP positive cells in a 1:1 ratio for 4 h before intracellular IFN- γ staining.

In vitro proteasome digestion

The synthetic peptides EVALSTTGEIPFYGKAIPLEAIKGG and EVALPTTGEIPFYGKAIPLEAIKGG ($\geq 95\%$ purity) were purchased from EMC, Tübingen, Germany and digested using the 20S proteasome assay kit complemented with human constitutive or immunoproteasome (Boston Biochem). In brief, 20 μ g of peptide were digested with 4 μ g of SDS-activated proteasome in a 500 μ l reaction. The reaction was stopped on indicated time points by adding 3 volumes of ice-cold acetone. For precipitation of proteasome the samples were subsequently frozen at -20°C for 30 min and then centrifuged for 30 min at 4°C with 15000 rpm. Supernatants were collected, evaporated to dryness and dissolved in 50 μ l 0.1 % trifluoroacetic acid. The peptide concentration of the resulting solution was determined by amino acid analysis as previously described (23). For the subsequent LC-MS/MS experiment, the sample was diluted to a concentration of 0.33 pmol/ μ l and 5 pmol were analyzed using an Ultimate 3000 RSLCnano system online coupled to a Velos Pro linear ion trap mass spectrometer (both Thermo Scientific, Bremen, Germany) as described earlier (23). The acquired raw files were further analyzed with Proteome Discoverer software (Thermo Scientific, ver. 1.3.0.339) and

searched with Sequest (24) against a self-written database containing the investigated pt and S1368P peptides. Precursor and fragment ion mass tolerance was set to 0.4 Da and the confidence level of peptide identification was set to a false discovery rate of 1 %. Relative peptide quantification was carried out via spectral counting. Thereto a normalized spectral index was calculated for each of the identified peptides by dividing the number of acquired peptide spectrum matches (PSMs) of a particular peptide by the number of PSMs acquired in the whole sample.

Generation and analysis of TNcc viruses.

Plasmid pTNcc encoding for the GT1a virus TNcc was kindly provided by Jens Bukh (25). Mutations S1368P, I1373V and I1380L were introduced by site directed mutagenesis and all constructs were verified by DNA sequencing. In vitro transcription and electroporation of Huh-7.5 cells was performed as described before (26). 4 h post transfection medium was changed with fresh DMEM (2 mM l-glutamine, nonessential amino acids, 100 U/ml of penicillin, 100 µg/ml of streptomycin, and 10 % fetal calf serum) supplemented with or without 10 mM 2'CMA (kindly provide by T. Tellinghuisen,(27)). 72 h post transfection, cell-free supernatant was filtered through 0.45 µM filters and 10x concentrated through Amicon centrifugal filters (Millipore). Virus titers were determined as described elsewhere (28) with slight modifications. In brief, Huh-7.5 cells were seeded in 96-well plates at a density of 1×10^4 cells per well 24 h prior to inoculation with dilutions of filtered cell culture supernatant (at least six wells were used per dilution). After 3 days, cells were washed with PBS, fixed for 20 min with ice-cold methanol at -20°C , washed three times with PBS. HCV infected cells were detected with anti-core C7.50 (1:300) (29) and anti-NS5A 9E10 (1:1000) (28) antibody in PBS for 45 min at room temperature. Cells were washed as described above, and bound antibodies were detected by incubation with

horseradish peroxidase-conjugated antibodies specific to murine IgG (Sigma-Aldrich, Steinheim, Germany) diluted 1:200 in PBS. After 1 h incubation at room temperature, cells were washed as specified above. Peroxidase activity was detected by using Carbazole substrate (0.32% (w/v) of 3-amino-9-ethylcarazole (Sigma) in N,N-dimethyl-formamide was diluted at a ratio of 1:3.3 with 15 mM acetic acid, 35 mM sodium acetate, pH 8.0, and 0.4% H₂O₂). Virus titers (50% tissue culture infective dose [TCID₅₀/ml]) were calculated based on the method of Spearman and Kärber. HCV RNA was quantified by X-tail reverse transcriptase (RT)-PCR as described before (22). For detection of HCV core protein virus containing supernatant was inactivated by addition of Triton X-100 to a final concentration of 1% (v/v) and the amount of released core protein was determined by the commercially available core ELISA ARCHITECT HCV core AG test (Abbott, Wiesbaden, Germany).

Statistical Analysis.

All statistical tests were performed using GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA).

Results

CD8⁺ T cells directed against the epitope IPFYGKAI₁₃₇₃₋₁₃₈₀ are detected after spontaneous resolution and in chronic genotype 3a infection but not in chronic genotype 1a infection

We previously reported an HLA-B*51-restricted epitope in NS3 (IPFYGKAI₁₃₇₃₋₁₃₈₀) that is >90% conserved across the HCV genotypes 1a, 1b and 3a (19). The CD8⁺ T cell immune response directed against the epitope IPFYGKAI₁₃₇₃₋₁₃₈₀ was analyzed in a cohort of anti-HCV positive PWID carrying the HLA-B*51 allele. A total of 43 subjects were analyzed, including 15 anti-HCV positive PWID with undetectable HCV-RNA, 13 PWID infected with GT1a, 3 PWID infected with GT1b and 12 PWID infected with GT3a. After 10 days of *in vitro* expansion IPFYGKAI-specific CD8⁺ T cells were detectable in 12 out of 15 (80%) individuals with undetectable viremia and in 6 out of 12 (50%) patients chronically infected with genotype 3a (figure 1A). In contrast, CD8⁺ T cells directed against this epitope were not detectable after antigen-specific expansion in patients with genotype 1a or 1b infection (figure 1A). Similar results were obtained when CD8⁺ T cell were directly analyzed *ex vivo* with HLA class I/peptide-dextramers. IPFYGKAI-specific CD8⁺ T cells were detectable in most HCV-RNA negative PWID and some PWID with genotype 3a infection. Notably, the four genotype 3a-infected patients with high frequencies were identical to those where antigen-specific CD8⁺ T cells were efficiently expanded *in vitro* (figure 1A). In turn, the analysis with HLA class I/peptide dextramers confirmed lack of detection of IPFYGKAI-specific CD8⁺ T cells in patients infected with genotype 1 (figure 1B). The CD8⁺ T cell response against the epitope IPFYGKAI₁₃₇₃₋₁₃₈₀ was also studied in a patient with acute HCV genotype 1a infection. The patient was previously reported in a study on the impact of HLA-B*57 on HCV infection outcome (30) and was also HLA-B*51 positive. Interestingly, here, the response was detectable by week 11 after infection but became completely undetectable in an IFN- γ ELISpot by week 47 (figure 1C).

The autologous virus of 22 PWID from the total of 26 with detectable HCV-RNA was sequenced. The sequences of the epitope and the flanking region are shown in table 1 and are indicated in figure 1A for PWID with genotype 3a infection with a detectable CD8⁺ T cell response (colored in figure 1A). In three of ten HCV genotype 3a sequences the virus harbored substitutions in the epitope region (I1373V, K1377R or I1380L). Of note, all three patients mounted robust responses against the prototype sequence (figure 1A). In functional assays the I1380L variant impaired recognition by antigen-specific CD8⁺ T cells whereas the I1373V variant was cross-reactive in HCV-RNA negative PWIDs and to a lesser extent in GT3a infected PWIDs (figure 1D and E). Notably, the three remaining GT3a-infected patients with detectable CD8⁺ T cell responses and no evidence for escape mutations had a history of injection drug use of less than 6 months consistent with more recent exposure and infection with HCV possibly at a stage prior to mutational escape. One of nine GT1a sequences and one of three GT1b sequences harbored the I1373V substitution in position 1 of the epitope. In both patients no CD8⁺ T cell response was detectable. One PWID with genotype 1a infection harbored a S1368P substitution. Notably, this substitution was also selected in the patient with acute HCV genotype 1a infection and with the rapid decline of the CD8⁺ T cell response (figure 1C and data not shown).

*The residue under selection pressure in HLA-B*51-positive individuals depends on the viral genotype*

Given the relatively high reproducibility of IPFYGKAI-specific CD8⁺ T cell responses in HCV-RNA negative PWID we aimed to address if there is evidence for immune escape in patients with persistent HCV infection at a population level. We therefore analyzed the impact of HLA-B*51-expression on the frequency of sequence polymorphisms in this epitope in different cohorts. In an

analysis of 442 genotype 1a sequences from a multi-center cohort (figure 2A) there was no evidence for mutational escape inside the epitope. Although an I1373V polymorphism was observed, this substitution was not enriched in HLA-B*51-positive patients. In contrast, the I1373V polymorphism was slightly enriched in HLA-B*51-positive patients from a multi-center cohort of 145 genotype 1b infected patients (figure 2B), although this difference was not statistically significant. However, in a large genotype 1b single source outbreak there was statistical evidence for selection of polymorphisms in position 1 (I1373V) and 8 (I1380L) of the epitope in the presence of HLA-B*51 (figure 2C). Finally, we also analyzed a local cohort of 102 patients infected with genotype 3a (figure 2D). Here, there was statistical evidence for selection of the I1373V polymorphism inside the epitope. Importantly, even though in genotype 1a there was no evidence for mutational escape inside the epitope, there was strong statistical evidence for HLA-B*51-associated selection pressure on a residue located five amino acids up-stream of the epitope (S1368) (figure 2A). In genotype 1a 20.7% of the HLA-B*51-positive patients carried a S1368P substitution, in contrast, only 0.8% of the HLA-B*51-negative patients carried any substitutions in this position ($p=0.00002$). Notably, in genotype 3a an S1369P substitution was also enriched in HLA-B*51-positive individuals, however, the difference was not statistically significant (figure 2D). Taken together, by analyses of different cohorts we found statistical evidence for mutational escape inside the HLA-B*51-restricted epitope (IPFYGKAI) in genotype 1b and 3a and evidence for selection of a distinct substitution in the epitope flanking region in genotype 1a.

*The S1368P substitution impairs targeting of the endogenously processed HLA-B*51 restricted epitope IPFYGKAI₁₃₇₃₋₁₃₈₀*

HCV genotype 1a was unique because evidence for HLA-B*51-associated selection pressure was

292 only observed in the epitope flanking region. Of note, this region does not contain a second HLA-
 293 B*51 binding motif by analysis with prediction algorithms for HLA class I-binding
 294 (www.immuneepitope.org). We therefore hypothesized that the S1368P may impair processing
 295 and presentation of the epitope. To address this experimentally, an assay that allows analysis of
 296 endogenously processed antigens was established. Effector CD8⁺ T cells directed against the
 297 HLA-B*51-restricted epitope IPFYGKAI were obtained by 10 days of antigen-specific
 298 expansion from different PWID with spontaneous immune control of HCV. To generate target
 299 cells that present the endogenously processed epitope HLA-B*51 positive buffy coat cells were
 300 transfected with expression plasmids encoding a fusion protein of a short fragment of NS3
 301 containing the epitope fused to GFP. 24h after transfection GFP-positive cells were sorted and
 302 used as targets for specific CD8⁺ T. As a positive control native target cells were pulsed with the
 303 synthetic peptide overnight. Figure 3A shows a representative result of one experiment. Upon
 304 stimulation with peptide-pulsed targets 6.3% IFN- γ ⁺ CD8⁺ T cells were detectable. When targets
 305 were transfected with a genotype 1a prototype sequence 7.3% of CD8⁺ T cells secreted IFN- γ . In
 306 contrast, when targets were transfected with the plasmid harboring the S1368P substitution the
 307 number of IFN- γ ⁺ CD8⁺ T cells was reduced to 1.3%. In seven independent experiments the CD8⁺
 308 T cell response against targets transfected with the S1368P-variant was reproducibly reduced to
 309 levels of 24% compared to prototype 1a (figure 3B). This suggests that less antigen was
 310 presented on S1368P-transfected target cells consistent with impaired endogenous processing
 311 associated with this substitution. To compare the processing efficiency between genotype 1a and
 312 genotype 3a the same fusion proteins were constructed with the 3a prototype sequence and the
 313 genotype 3a S1369P substitution. In three independent experiments the CD8⁺ T cell response
 314 against targets transfected with prototype 3a were reproducibly weaker (33%) compared to
 315 prototype 1a. The S1369P substitution further reduced the response to 6% compared to prototype

1a. This suggests that the epitope is less efficiently processed in a genotype 3a context compared to a genotype 1a context. Moreover, both substitutions in the epitope flanking region (S1368P in genotype 1a and S1369P in genotype 3a) further reduce the antigen processing efficiency.

Differential proteasomal cleavage of peptides with the S1368P substitution

It was next addressed if the S1368P substitution selected in genotype 1a has an impact on proteasomal cleavage consistent with altered processing. Therefore, synthetic peptides 25 amino acids in length either with the prototype sequence (pt) or harboring the S1368P substitution (S1368P) were digested with constitutive or immune proteasome and the cleavage products were analyzed by mass spectrometry. In figure 4 the normalized spectral indices for cleavage products containing the full epitope sequence are shown at different time points after digestion with the constitutive proteasome (figure 4A) or the immune proteasome (figure 4B). There was no significant difference in the relative frequency of epitope containing peptides upon digestion of the prototype or the S1368P variant at any time. In fact, there was a minor trend towards higher frequencies of epitope containing peptides after 24h when the S1368P variant was digested (figure 4A and B). As carboxypeptidase activity is basically absent from the endoplasmatic reticulum (ER), antigen presentation requires peptides with correct C-terminal ends after proteasomal cleavage (31). We therefore focused the analysis on peptide products ending with the epitope sequence IPFYGKAI. Figure 4C and D show the normalized spectral indices of individual peptides after digestion by the constitutive proteaseome (figure 4C) or the immune proteasome (figure 4D) at different time points. After digestion of the prototype peptide the most prevalent cleavage product was STTGEIPFYGKAI carrying the residue that was under selection pressure in HLA-B*51-positive patients in genotype 1a at its N-terminal end (boxed in figure 4C and D). Also highly prevalent were N-extended peptides with three (TGEIPFYGKAI) or four

(TTGEIPFYGKAI) additional residues. When the S1368P variant was digested the most prevalent cleavage product (PTTGEIPFYGKAI) carried the substitution selected in HLA-B*51-positive patients in genotype 1a at its N-terminal end. Importantly, shorter N-extended cleavage products with three or four additional residues were nearly absent after digestion of the S1368P variant. Similar results were obtained after digestion by the constitutive proteasome (figure 4C) or the immune proteasome (figure 4D).

The S1368P substitution does not impair viral fitness in HCV genotype 1a

We hypothesized that fitness constraints caused preferred selection of the S1368P substitution in genotype 1a. To address this experimentally, the recently described genotype 1a full genome virus TNcc (25) was utilized to study the replication fitness and infectivity associated with the substitutions S1368P, I1373V or I1380L. 72h after electroporation of virus RNA into Huh-7.5 cells supernatants were used for infection of naïve Huh-7.5 cells (figure 5A). Upon transfection with the parental HCV TN genome the TCID₅₀ was 1.1×10^4 infectious particles per ml. Approximately the same amount of infectious particles was released upon transfection with the S1368P variant (0.8×10^4 TCID₅₀/ml) or the I1373V variant (1.3×10^4 TCID₅₀/ml). In contrast, the I1380L substitution was associated with a decreased TCID₅₀ of 0.2×10^4 per ml. The same hierarchy was observed at the replication level, with only a slight reduction of replication for the S1368P and I1373V substitution and a 3-fold reduced replication for the I1380L substitution. This indicates that only the substitution I1380L impairs viral fitness in genotype 1a whereas the substitutions S1368P and I1380L do not.

Discussion

Here, we characterized a highly conserved HLA-B*51-restricted CD8⁺ T cell epitope located in

the helicase domain of HCV NS3. The high frequency of CD8⁺ T cells specific for this epitope in PWID who achieved spontaneous immune control of HCV infection suggests that this CD8⁺ T cell response is reproducibly mounted in HLA-B*51-positive patients. Given the high degree of conservation of the epitope, it was therefore surprising that in chronic infection CD8⁺ T cell responses against this epitope were detectable only in PWID infected with genotype 3a and were completely absent in patients infected with genotype 1a. Although viral sequence polymorphisms were overall rare, there was selection pressure on the epitope containing region as evidenced by HLA-B*51-associated viral sequence polymorphisms in all studied genotypes. Notably, in genotype 1b there was statistical support for selection of escape mutations inside the epitope in the single-source outbreak, whereas there was only a non-significant trend in the genotype 1b multi-center cohort. It is possible that escape patterns inside this epitope differ between cohorts. Alternatively, because the natural sequence variability in this epitope in the genotype 1b multi-center cohort is higher compared with the single-source outbreak, the size of the multi-center cohort may be too small to unmask an existing effect of HLA-B*51-associated selection pressure.

The predominant escape mechanisms of substitutions selected by CD8⁺ T cell immune pressure in HCV epitopes are impaired binding of the variant epitope to the HLA class I-molecule or impaired binding of the T cell receptor to the HLA class I/peptide-complex. As an alternative mechanism, processing of the variant epitope can be affected by substitutions in the epitope region. We previously reported CD8⁺ T cell selection of a substitution inside the HLA-B*08-restricted epitope NS3 HSKKKCDEL₁₃₉₅₋₁₄₀₃ located in position 9 of the epitope (9). Functional analyses revealed that the selected leucine to valine or phenylalanine substitution impaired presentation of the endogenously processed epitope. Similarly, Kimura et al. (32) described substitutions selected inside targeted CD8⁺ T cell epitopes that impaired proteasomal processing

in the chimpanzee model. In contrast, CD8⁺ T cell selection of substitutions in the epitope flanking region has not been described yet in HCV. Seifert et al. (33) demonstrated that a tyrosine to phenylalanine polymorphism located in the C-terminal position +1 of the HLA-A*02-restricted epitope NS3 CVNGVCWTV_{1073–1081} influenced carboxyterminal cleavage of the epitope by the proteasome, however, there was no conclusive evidence for immune selection pressure on this residue (22, 33). In HIV larger population studies of HLA class I-associated viral adaptation suggested that selection of processing mutations in the epitope flanking region contributes to mutational immune escape (34). This included substitutions in the C-terminal and N-terminal epitope flanking regions. Only a few studies directly addressed the functional consequences of such putative processing mutations (35, 36). The example of selection of altered processing in HLA-B*51-positive patients infected with HCV genotype 1a supports that similar immune escape strategies are utilized by HCV.

Processing and presentation of viral epitopes is a multistep process starting with production of peptide precursors by proteasomal degradation of viral proteins (reviewed in (37)). These peptide precursors undergo N-terminal trimming by different endopeptidases and aminopeptidases in the cytosol and by the aminopeptidases ERAP1 and ERAP2 after the peptides have been transported by TAP to the ER (38, 39). Importantly, since carboxypeptidase activity is absent from the ER, production of peptide precursors with correct C-terminal ends by the proteasome is required for subsequent HLA class I-presentation (31). It has been highlighted that the trimming efficiency of N-extended epitopes strongly depends on the amino acid composition of the epitope flanking region. Some amino acids such as leucine, lysine, phenylalanine and methionine are cleaved with high activity whereas amino acids such as proline and glutamic acid are only poorly cleaved (40). Accordingly, these latter amino acids are underrepresented in flanking regions of known CD8⁺ T

cell epitopes (41). In HCV genotype 1a there was HLA-B*51-associated selection pressure on position -5 of the epitope with a substitution from serine to proline. Digestion of the N- and C-extended variant peptide by the constitutive and the immune proteasome yielded predominantly precursor peptides starting with proline, whereas digestion of the prototype yielded also shorter N-extended precursor peptides. The shorter peptides are advantageous for the transport into the ER (42), and more importantly, N-terminal trimming of the peptide precursor starting with proline is inhibited (31). This suggests that the serine to proline substitution altered proteasomal production of epitope precursors with the consequence of a very low abundance of cleavable N-extended epitopes in the ER, although the latter was not formally tested.

Selection of CD8⁺ T cell escape mutations is a trade-off between functional escape and viral constraints. Indeed, HCV may explore its replication space with different substitutions in targeted epitopes before some mutations reach fixation (9, 43). The example presented here underlines that even despite an epitope being conserved across HCV genotypes the selected escape pathways and the CD8⁺ T cell response can substantially differ. Although not fully conclusive, our data suggest that selection of the S1368P substitution represents the optimal trade-off between functional immune escape and viral fitness in genotype 1a. The I1380L substitution may be disadvantageous due to its fitness costs despite efficient immune escape. In turn, the degree of cross-reactivity of the I1373V variant may prevent fixation in genotype 1a despite its low fitness costs. Interestingly, we observed inter-individual differences in the level of cross-reactivity of the I1373V substitution with high cross-reactivity in HCV-RNA negative PWIDs and lower levels of cross-reactivity in PWIDs infected with genotype 3a (figure 1D and E) suggesting that the degree of cross-reactivity may influence the outcome of infection as previously reported (17). Absence of the CD8⁺ T cell response in PWID with chronic genotype 1a infection is unlikely caused by

lack of priming. The rapid decline of the response in the patient with acute HCV genotype 1a infection rather suggests secondary failure. This patient harboured a virus with the S1368P substitution. Although the CD8⁺ T cell response was clearly impaired against endogenously processed antigens carrying the S1368P substitution, there was still some degree of T cell reactivity in our assays. This residual epitope presentation may cause continuous CD8⁺ T cell stimulation associated with progressive dysfunction or exhaustion of antigen-specific CD8⁺ T cells (44-46) and possibly ultimate extinction of these cells (46).

The degree of CD8⁺ T cell reactivity against the S1368P substitution in the context of genotype 1a was comparable to the reactivity against the endogenously processed genotype 3a antigen suggesting that the epitope is less efficiently processed in the context of genotype 3a. Notably, in genotype 3a the substitution S1369P also impaired recognition of the endogenously processed antigen, however, here the I1373V was preferentially selected. One possibility is that in combination with the lower processing efficiency of the epitope in genotype 3a the I1373V substitution is sufficient to escape from the immune response. A second plausible explanation is that the I1373V is associated with lower fitness costs compared to S1369P and I1380L in genotype 3a. However, this cannot be addressed until robust genotype 3a infectious viruses are publically available. In either case, at least in some individuals the corresponding CD8⁺ T cell response was preserved in our cohort of PWIDs infected with genotype 3a consistent with a memory T cell phenotype after mutational escape of the targeted antigen as previously reported (44, 45).

Taken together, HCV is able to evolutionary adapt to CD8⁺ T cell immune pressure in multiple ways. The pathways to mutational escape are predictable but are distinct in different genotypes.

Beyond selection of mutations inside targeted epitopes that impair HLA class I binding of the variant peptide ligand or TCR binding to the variant HLA class I/peptide-complex HCV also inhibits epitope processing by modification of the epitope flanking region under T cell immune pressure. Importantly, the selected escape pathway of HCV may have consequences for the destiny of antigen-specific CD8⁺ T cells.

Acknowledgements

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Figure legends**Figure 1: CD8⁺ T cell response against the epitope IPFYGKAI₁₃₇₃₋₁₃₈₀ in PWID exposed to**

HCV. (A) T cells were expanded for 10 days from PBMCs in the presence of the peptide IPFYGKAI. After *in vitro* expansion cells were re-stimulated with the peptide before intracellular IFN- γ staining. The autologous viral epitope sequences from GT3a infected PWID with detectable responses are indicated. (B) HLA-B*51₁₃₇₃₋₁₃₈₀ specific T cells were detected *ex vivo* via IPFYGKAI-specific HLA-B*5101 dextramer staining. (C) The HLA-B*51₁₃₇₃₋₁₃₈₀ specific T cell response was determined by ELISpot at the indicated time points in a patient with acute HCV infection. (D&E) Serial peptide dilutions of the prototype (green), I1373V (blue) or I1380L (red) sequence of the B51-1373 peptide were tested in RNA negative (D) or GT3a infected (E) patients. Statistical comparisons between groups in (A) and (B) were done with a Kruskal-Wallis test and significant p-values are indicated.

Figure 2: Frequency of HLA-B*51-associated viral polymorphisms in the epitope region.

The frequency of variations from the reported prototype sequence of the epitope region in GT1a (A), GT1b (B), the East-German Anti-D cohort (C) and in GT3a (D) are shown for patients carrying the HLA-B*51 allele (red) and patients not carrying the HLA-B*51 allele (green). Positions with significant differences in polymorphism frequencies in the absence and presence of HLA-B*51 are marked and the p-values (Fisher's exact test) and the most frequent variant amino acid are indicated.

Figure 3: CD8⁺ T cell response against the endogenously processed epitope IPFYGKAI₁₃₇₃₋

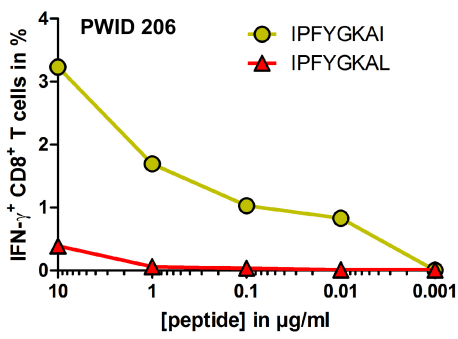
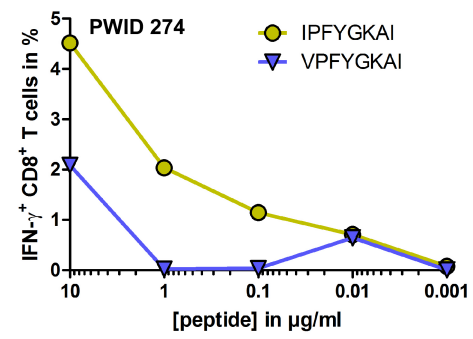
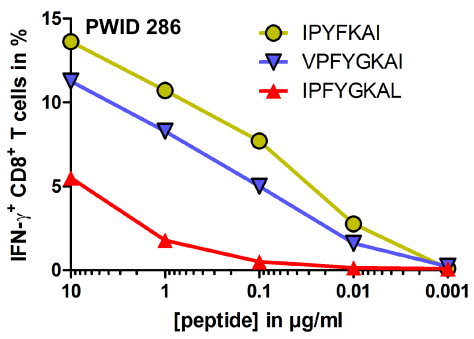
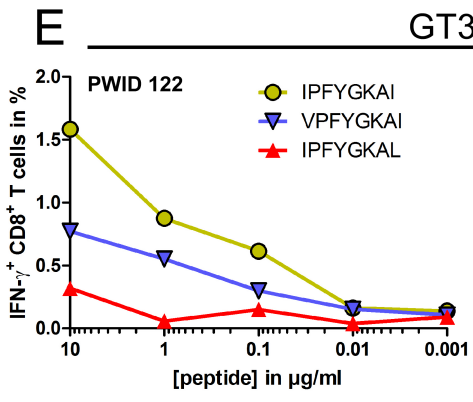
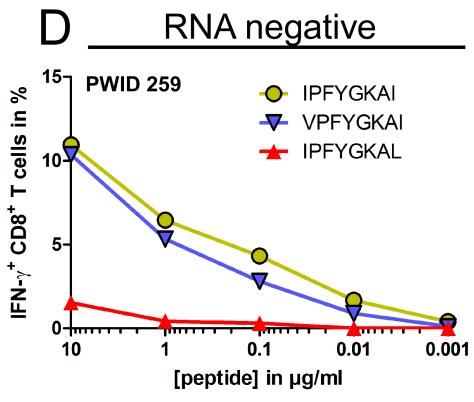
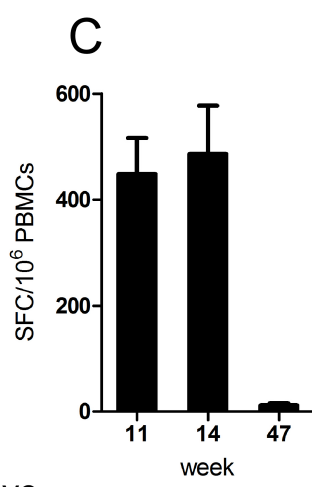
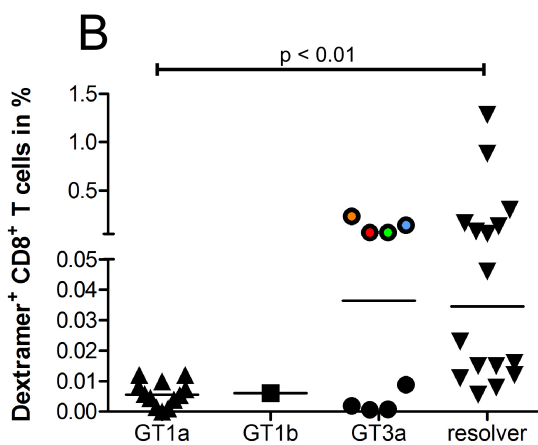
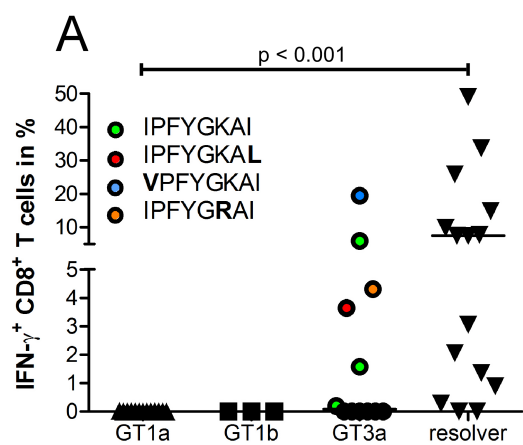
1380. Effector T cells were expanded for 10 days from PBMCs in the presence of the peptide IPFYGKAI. HLA-B*51-positive target cells were generated by electroporation with a

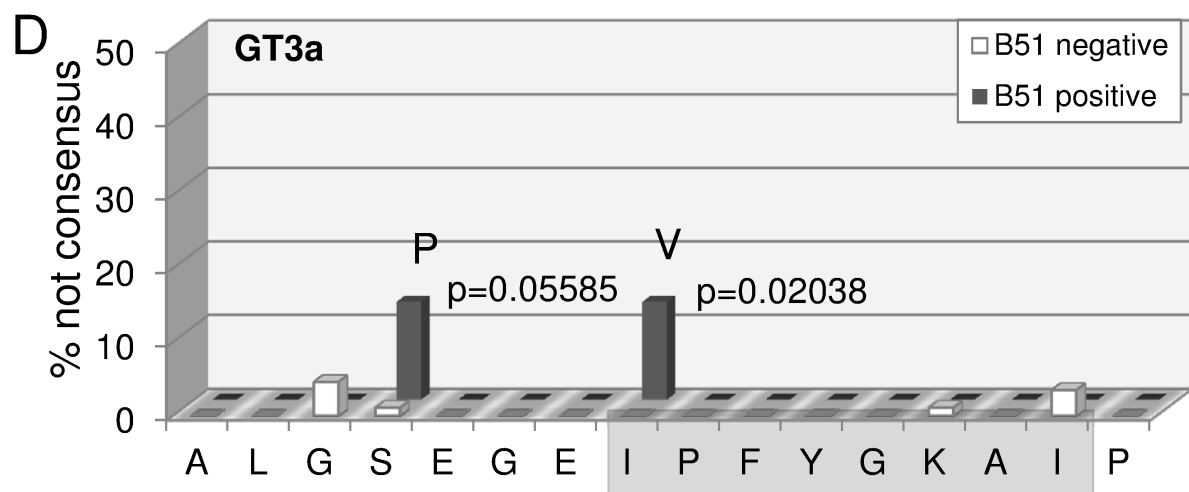
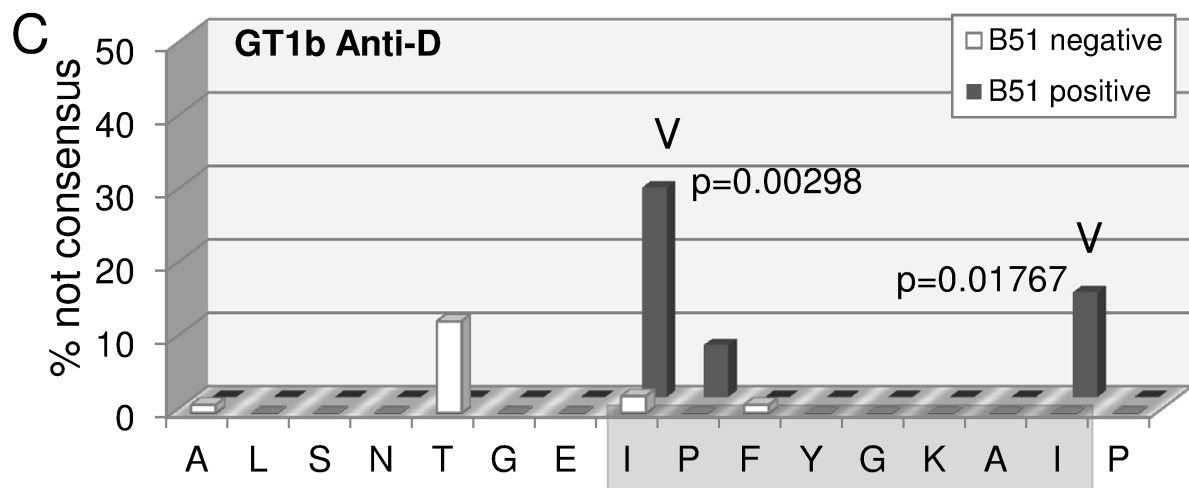
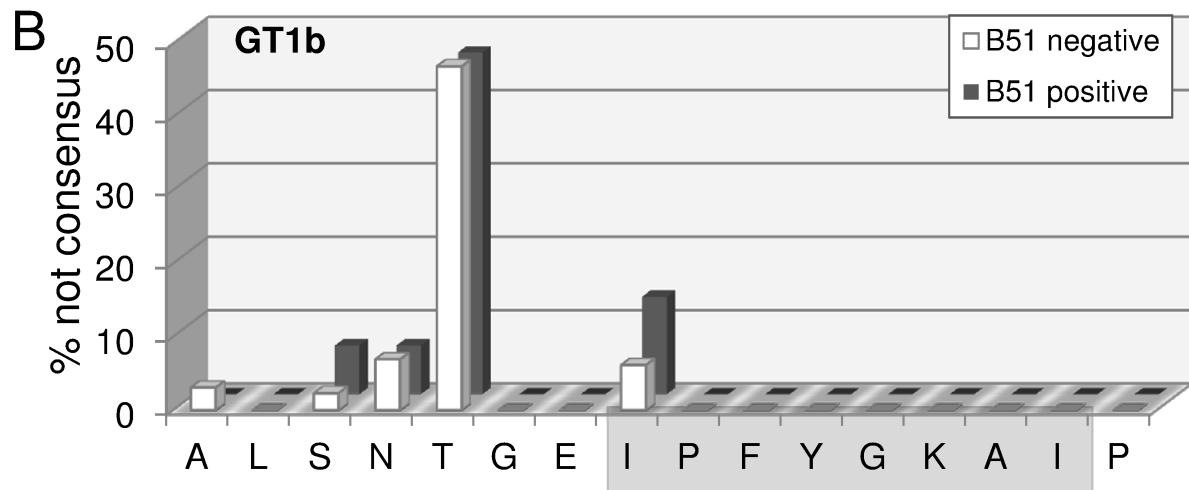
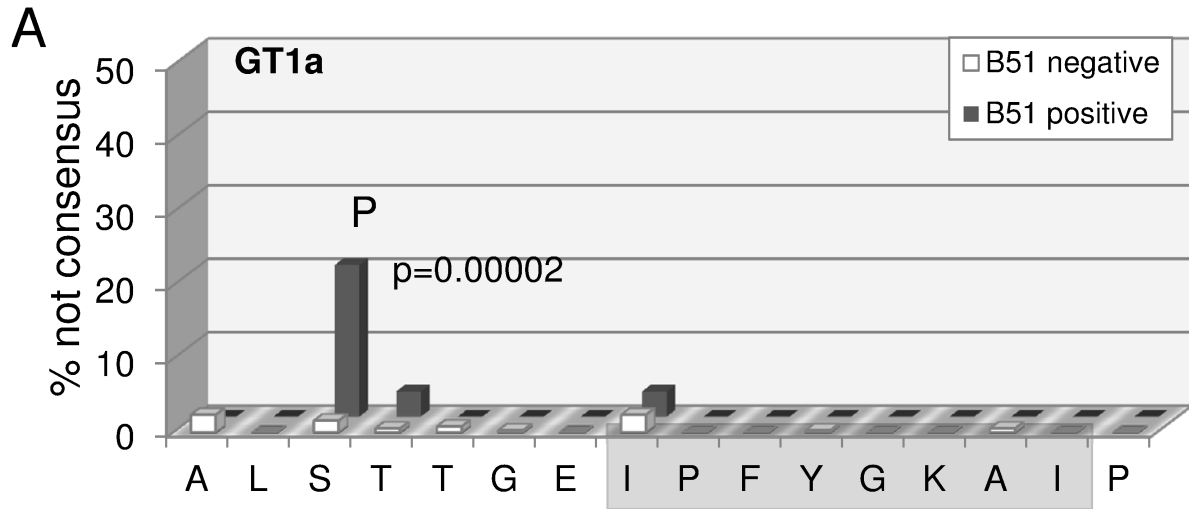
NS3(aa1330-2420)-GFP fusion protein. GFP positive cells were sorted and used as targets for restimulation of IPFYGKAI-specific effector CD8⁺ T cells in an effector:target ratio of 1:1 for 4h followed by an ICS. Mock transfected targets and peptide-pulsed targets served as negative or positive controls, respectively. (A) Representative FACS results of one ICS. (B) The IFN- γ response against targets transfected with GT1a prototype NS3 was normalized to 100% and compared to the response against other targets as indicated. Data represent results from at least three independent experiments. P-values was calculated using a one sample t test with a hypothetical value of 100 (S1368P versus pt1a) or an unpaired t test (pt GT3a versus S1369P).

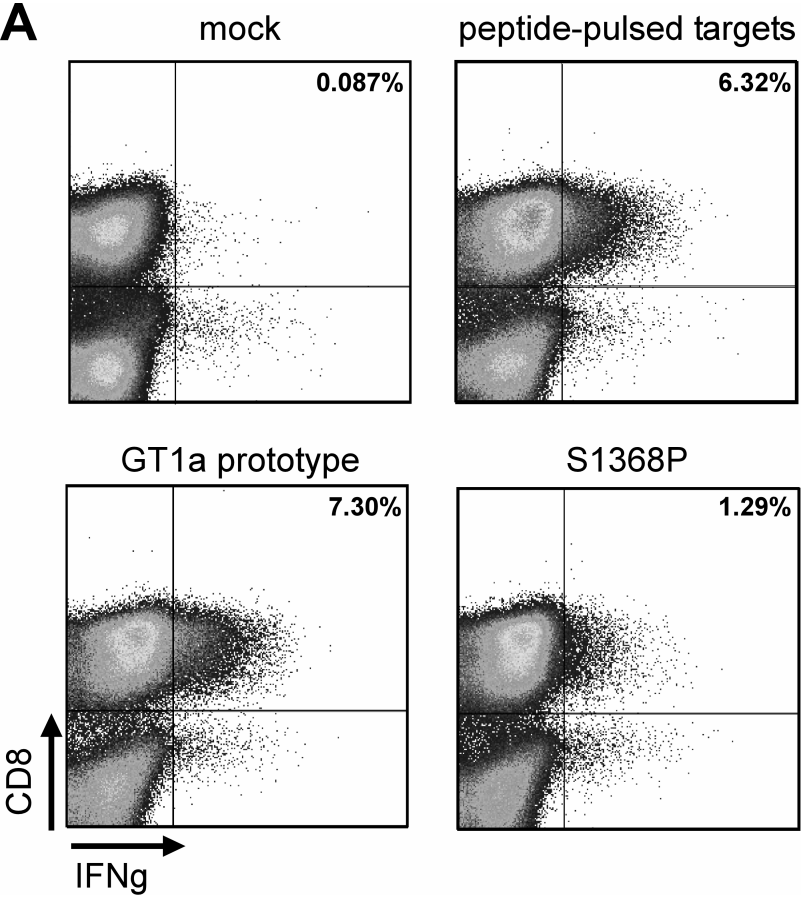
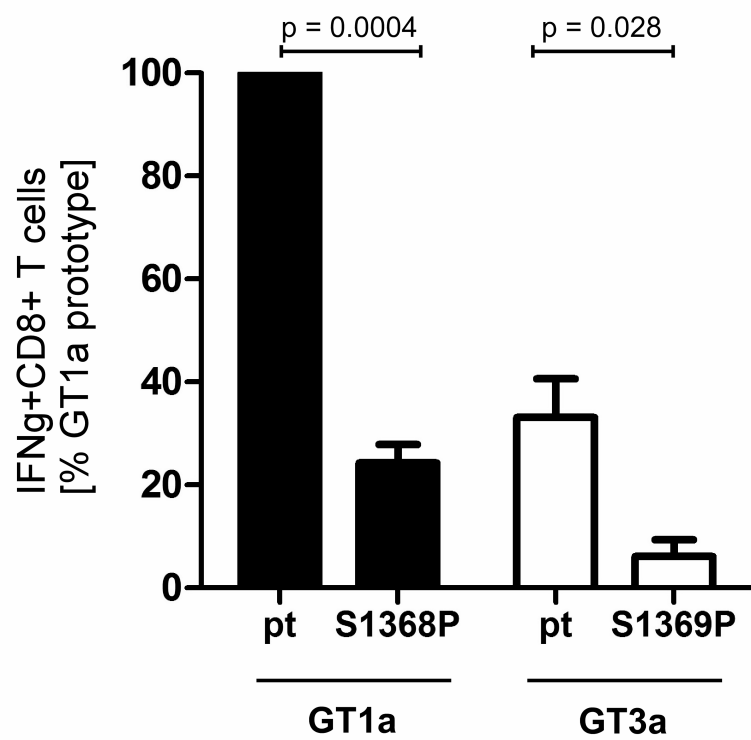
Figure 4: Impact of the S1368P substitution on proteasomal degradation. (A and B) The relative abundance of epitope-containing cleavage products after digestion of 25mer peptides with the prototype sequence (pt) or carrying the S1368P substitution (S1368P) with (A) constitutive proteasome or (B) immune proteasome is shown by normalized spectral indices. (C and D) The relative abundance of cleavage products with the correct C-terminal epitope end is shown by normalized spectral indices of individual peptides after digestion with (C) constitutive proteasome or (D) immune proteasome.

Figure 5: Infectivity and Replication of wild type and mutant TNcc strains. RNA transcripts of the parental HCV TN genome and TN mutants were transfected into Huh-7.5 cells. (A) Seventy-two hours later, cell-free supernatant was used for inoculation of naïve Huh-7.5 cells. The TCID₅₀ of the variants was determined by a limiting dilution assay and staining with Core and NS5A-specific antibodies. (B) RNA levels 72h post-transfection were measured by quantitative real-time RT-PCR. The RNA levels in presence of 2'Methyladenosin (2'CMA) was

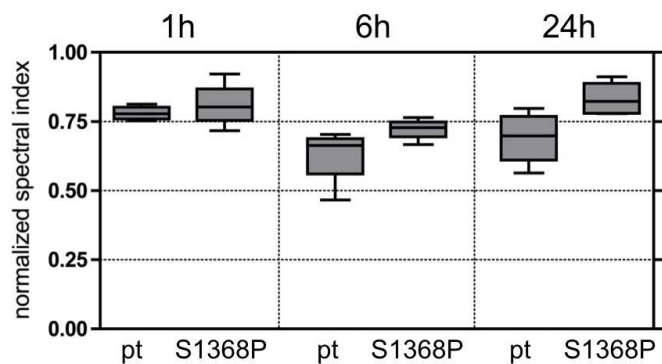
686 used for normalization. The ratio of measured HCV RNA to measured RNA levels in presence of
687 2'CMA was calculated for each construct. Data from four independent experiments are shown.
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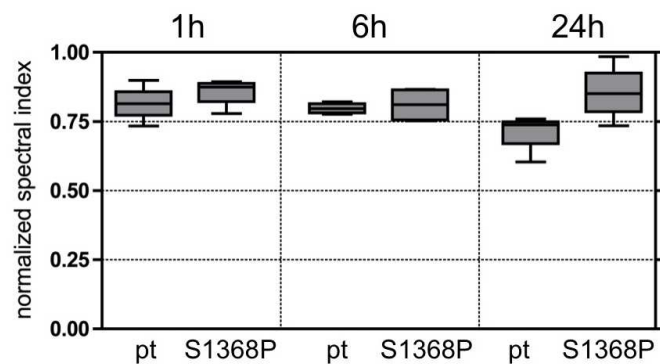


A**B**

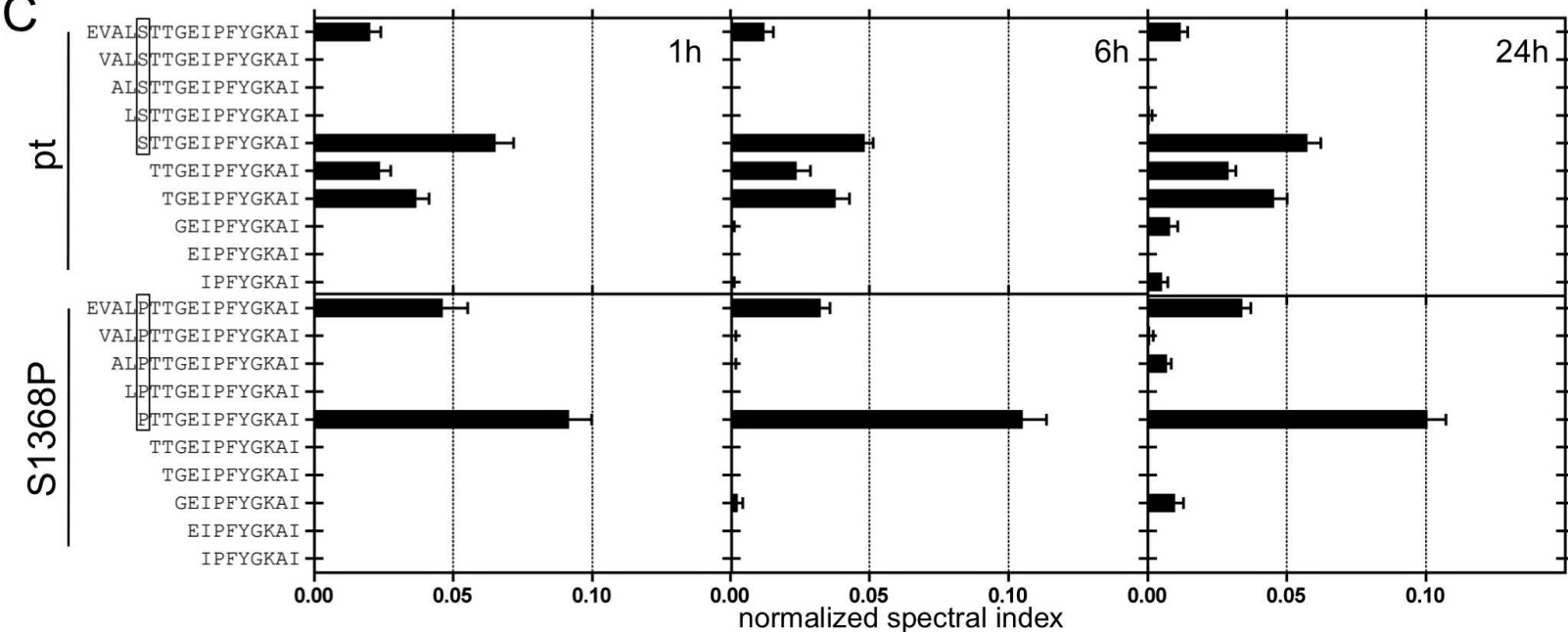
A



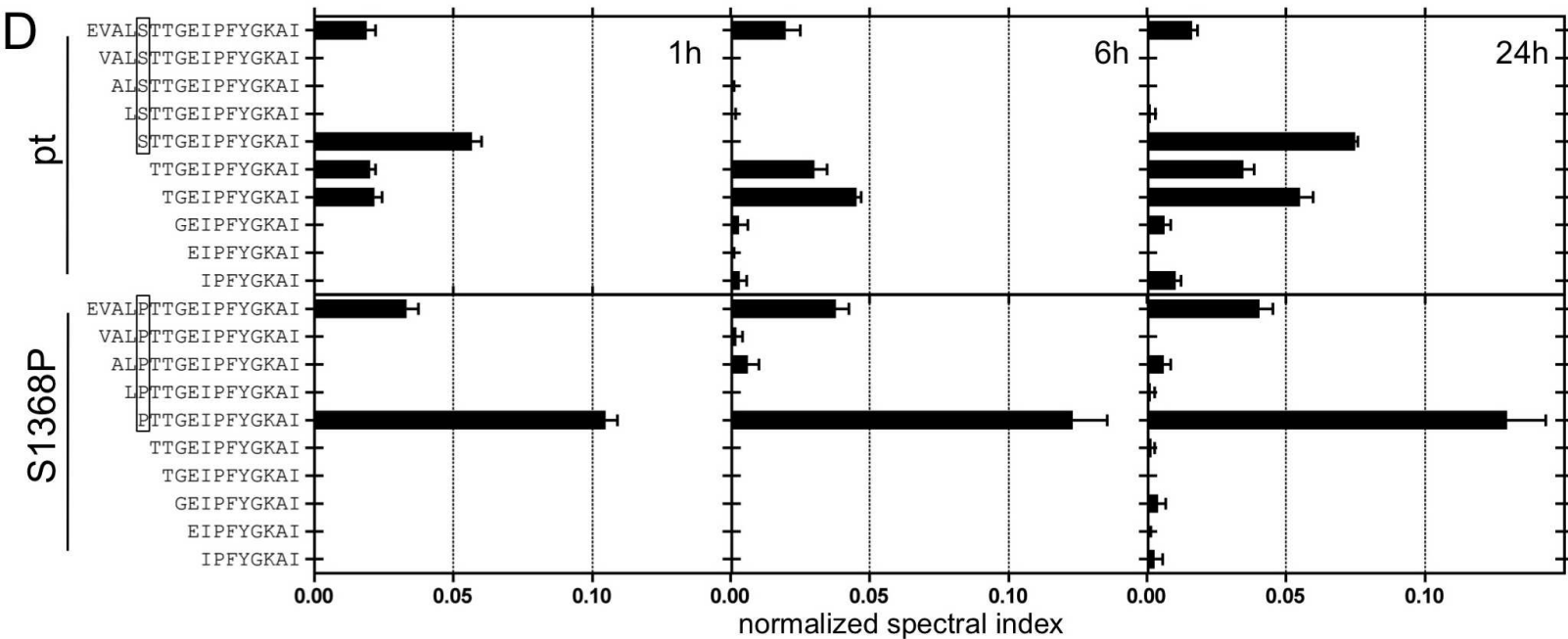
B



C



D



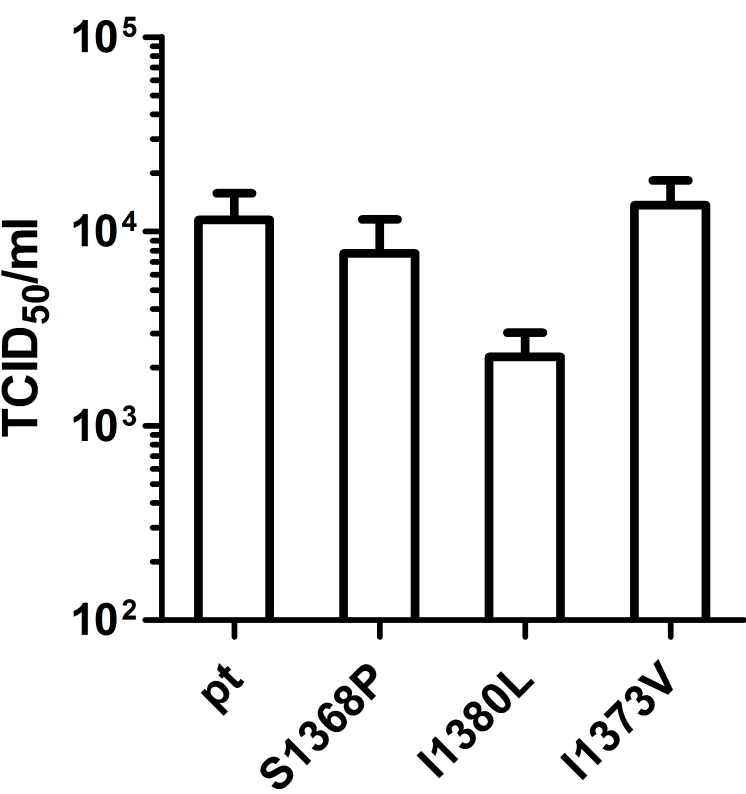
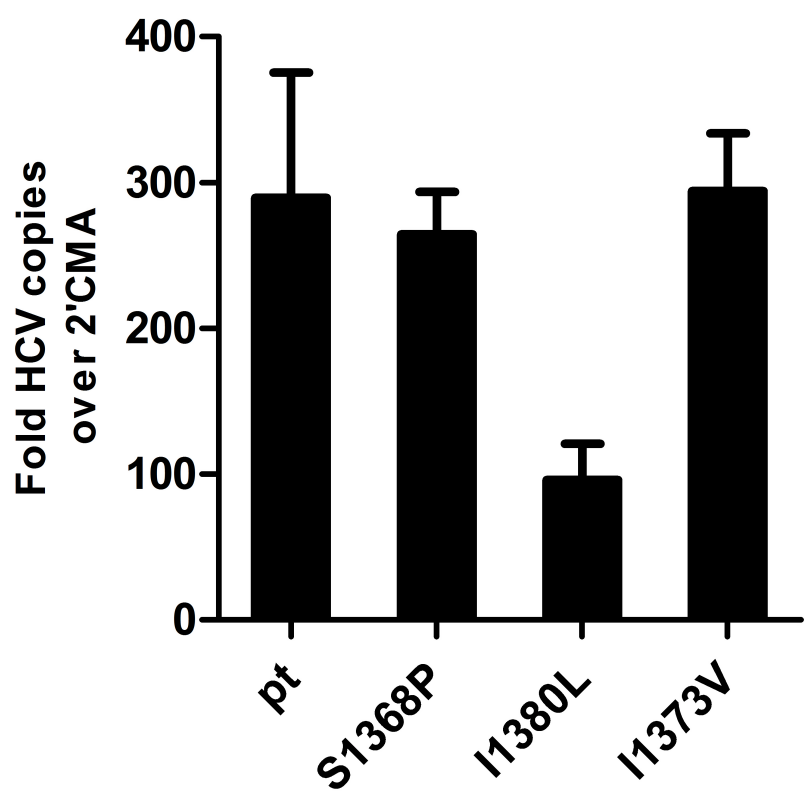
A**B**

Table 1: Patient characteristics

ID	Genotype	viral load IU/ml	A	L	S	T	T	G	E	I	P	F	Y	G	K	A	I	P	^a
138	1a	2148000
154	1a	777200
175	1a	2200000	.	.	P
251	1a	61790	not done																
278	1a	3015000	V
283	1a	2422000
299	1a	275700
324	1a	2469000
348	1a	83080
393	1a	5282000	not done																
408	1a	1562000
423	1a	320900	not done																
581	1a	3942000	not done																
084	1b	61550	.	.	.	N	I
117	1b	1033000	.	.	.	N	I	.	V
332	1b	220000	.	.	.	N
096	3a	247700	.	.	G	S	E
113	3a	2258000	.	.	G	S	E
122	3a	87400	.	.	G	S	E
137	3a	1046000	.	.	G	S	E
176	3a	924700	.	.	G	S	E
206	3a	368000	.	.	G	S	E	L	.	.
240	3a	154500	.	.	G	S	E
257	3a	3642	not done																
274	3a	146300	.	.	G	S	E	.	V
292	3a	80690	not done																
466	3a	34010000	.	.	G	S	E
533	3a	511500	.	.	G	S	D	R
042	RNA neg	-																	
062	RNA neg	-																	
110	RNA neg	-																	
161	RNA neg	-																	
196	RNA neg	-																	
242	RNA neg	-																	
264	RNA neg	-																	
286	RNA neg	-																	
344	RNA neg	-																	
365	RNA neg	-																	
417	RNA neg	-																	
418	RNA neg	-																	
474	RNA neg	-																	
574	RNA neg	-																	
587	RNA neg	-																	

^a H77 prototype sequence